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HISTOPATHOLOGICAL CHANGES IN GASTROCNEMIUS MUSCLES OF RABBITS INJECTED WITH HI – 6 IN SALINE (U)

by

C.E. Connolley-Mendoza, K. Jericho[1]
and T. Bhatti

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ABSTRACT

The gastrocnemius muscles of rabbits were injected with HI-6 in saline. Macroscopic and histopathological examinations of injection sites and regional lymph nodes revealed that HI-6 in saline produced muscle necrosis. Macroscopic examinations of muscles injected with a low dose of HI-6 (50 mg/kg) showed no lesions on Day 7. However, histopathological examinations disclosed lesions on some animals but with evidence of healing processes by Day 7; lesions disappeared by Day 14. Further macroscopic and histopathological examinations revealed that lesions associated with the high dose (200 mg/kg) were still prominent on Day 14 but with evidence of healing. Similar lesions seen in muscles injected with saline were significantly less persistent than those associated with HI-6. KEYWORDS: OXIMES, [illegible], ANTIDOTES. (Canada)

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INTRODUCTION

HI-6* is a potential therapeutic drug against chemical warfare agent poisoning, being particularly efficacious against soman (GD) (Clement, 1981). Lundy and Tremblay (1979) demonstrated its anti-muscarinic, antinicotinic and ganglionic blocking activities. Clement (1981) presented comprehensive data on the antidotal properties of this compound and he showed it to be the least toxic of these types of oximes. Boskovic (1981) and Wolthuis and coworkers (1981) further studied the pharmacological actions of this chemical. In most of these studies, HI-6 was injected by the intraperitoneal route. Studies on tissue damage due to HI-6 at the injection site in muscle are not available in the literature. In human use, the anticipated route of

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* [1-(((4-(aminocarbonylcarbonyl)pyridinio)methoxy)methyl-2-(hydroxy-imino)methyl) pyridinium dichloride]

administration of HI-6 is by the intramuscular route. Therefore, the histopathological changes in the rabbit muscle injected with HI-6 in saline were investigated in an attempt to determine if significant or irreversible muscle damage would occur.

METHODS

Test Materials

Purified , white and crystalline HI-6 (DRES batch 32) was used. The dosing solutions were prepared by dissolving HI-6 in sterile physiological saline (Travenol-Baxter, Mississauga, Ontario, Canada). Two HI-6 solutions, 63 and 250 mg/mL, were prepared immediately before use. These solutions were injected at dosages of 50 and 200 mg/kg body weight, respectively, at an injection rate of 0.8 mL/kg. The pH values of the solutions were as follows: saline, pH 5.4; HI-6 at 63 mg/mL, pH 3.5; and at 250 mg/mL pH 3.0.

Preparation of Animals

Mature, male rabbits Oryctolagus cuniculus (Maple Lane, Clifford, Ontario, Canada), a New Zealand white strain, weighing between 2.7 and 3.3 kg were used in these studies. A total of 36 rabbits were weighed, and randomly numbered and assigned to three treatment groups (Table 1). Before intramuscular injection, a skin area at the region of the gastrocnemius muscle was clipped with an electric shaver and then swabbed with alcohol. In each test group, the hindleg assigned for HI-6 injection was randomly selected while the contralateral leg served as a control. The control or test muscle received 0.8 mL/kg of saline or HI-6 solution, respectively. In addition, a group of control rabbits (n = 6) received the same volume

of saline on each gastrocnemius muscle. The injection areas were marked with non-toxic indelible ink.

Treatment of Animals

On Day 0, the day of treatment, saline or HI-6 in saline was injected into the gastrocnemius muscle via a 26 gauge needle. The dosages used are shown in Table 1. During injection, the solution was filter-sterilized using a 0.45 μm filter (Acrodisc ™, Gelman Sciences, Ann Arbor, Michigan, USA).

Throughout the study, animals were caged individually and placed in a room with a 12 h light and dark cycle. The animals were fed and given water ad lib. and were observed each day for clinical signs. Animals were weighed before they were killed by an overdose of thiopental (sodium pentothal) followed by exanguination. The number of rabbits killed per treatment group on Days 3, 7 and 14 are also shown in Table 1.

Tissue Examination

Tissue samples were taken from the injection sites in the muscle and from the heart, liver, kidney, spleen, and popliteal lymph node of each animal. They were immersed in 10% formalin in neutral saline solution buffered with 0.2 M sodium phosphate (Gomori's buffer). After fixation, tissues were trimmed and embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin (HE). Lesions in muscles were difficult to discern in fixed tissues, indicating that the trimming process may have missed the lesion center. For this reason, the size of histological lesions was not measured.

Well-defined lesions, visualized macroscopically as hemorrhagic or pale tissue, were measured with a ruler. The macroscopic and microscopic changes were described by a pathologist (K.E.J.) who was unaware of the identity of the specimen.

Statistical Methods

Sizes of the macroscopic lesions were compared among groups by using the Student's t test.

RESULTS

Daily observations of all animals in their cages revealed normal behaviour. The body weights and daily weight gains of animals from various treatment groups were not significantly different ($P \leq 0.05$) from each other (data not shown). One animal in the 50 mg/kg group which died on Day 6 failed to reveal any notable lesions at necropsy.

Macroscopic Changes

Muscular lesions were marked by either haemorrhage or pale tissue. Some lesions were prominent and well-defined whereas others were mild and poorly demarcated. Well-defined lesions were measurable at the 200 mg/kg HI-6 dosage on Days 3, 7 and 14, and in the control saline groups on Days 3 and 7 (Table 2). The macroscopic lesions seen in the 50 mg/kg groups on Day 3 were mild and not measurable. Muscles injected at this dosage were without lesions on Days 7 and 14. Two of the 15 muscle sites injected with saline had measurable lesions of 1 and 1.5 cm in diameter. Eleven of 15 muscle sites injected with HI-6 in saline had measurable lesions 1 to 3 cm in diameter.

Statistics

Comparison among lesions in saline-injected muscles (total n = 41) failed to reveal any significant differences (Table 3). However, the lesions observed in the 200 mg/kg HI-6 groups were significantly larger than those in either the 50 mg/kg HI-6 or saline group on Days 3 and 7 ($p \leq 0.01$).

Microscopic Changes

Muscle Injected with Saline Only

Three days after saline injection, 7 of 13 muscle sites examined showed necrosis, and infiltration by mononuclear cells and macrophages in the process of myophagia (Fig. 1). Seven days after saline injection, only 1 of 14 sites revealed these histopathological changes. Lesions were not seen 14 days after injection with saline.

Muscles Injected with HI-6 in Saline

Microscopic changes were seen on Day 3 to Day 14 in all muscles injected with 200 mg/kg of HI-6. Lesions studied 3 days after injection were quantitatively similar to saline-induced changes. Some lesions had haemorrhage and/or congestion, but this was not a constant feature (Fig. 2). Seven days after injection with the high dose (200 mg/kg), the lesions examined showed peripheral congestion, haemorrhage, central ischemic necrosis and macrophages in the process of myophagia. Mononuclear cells infiltrated the lesions but these were less numerous on Day 7 than on Day 3. Fourteen days after injection with the high dose, healing was evident in that haemorrhage and congestion were much reduced, fibroblasts had infiltrated the connective tissue, and "giant

cells" had formed (Fig. 3). In the sites injected with a low dose (50 mg/kg), lesions (2 of 5 injection sites) showed signs of repair on Day 7 but were still evident on Day 14.

Although the size of lesions were not measured microscopically, at 7 days, those produced by the low dose of HI-6 in saline appeared smaller than those produced by the high dose.

Lymph Nodes

Changes in the regional popliteal lymph nodes were only seen 7 and 14 days after injection with either saline or HI-6 (Table 2). The node, which received lymph from muscles injected with 200 mg/g of HI-6, showed 3 necrotic foci 7 days after injection (Fig. 4). Fourteen days after injection with the same dosage, 1 node showed focal necrosis of its capsular connective tissue.

On Day 7, 7 of 23 nodes showed a slight increase in polymorphononuclear cells; this increase was seen in nodes which received lymph from the area of sites injected with either saline or HI-6. On Day 14, 6 of 21 nodes showed a slight depletion of lymphoid tissue; this change was seen in nodes which received lymph from legs injected with either saline or HI-6.

Relationship of Macroscopic to Microscopic Changes

Table 2 also shows the relationship of the macroscopic to microscopic changes in injection sites. In muscles injected with 200 mg/kg of HI-6, macroscopic lesions were confirmed by histological examination of injection sites except for one site on Day 7 and two sites on Day 14. In muscles injected with 50 mg/kg, all macroscopic

lesions showed corresponding microscopic changes on Day 3. On Day 7, microscopic changes seen in 2 muscles which did not show macroscopic lesions, and on Day 14, neither macroscopic nor microscopic lesions were evident. In muscles injected with saline only (41 sites), mild macroscopic lesions at 2 sites could not be verified histologically while 2 sites with microscopic changes were without macroscopic changes.

Other Tissues

Histopathological examinations of heart, liver, kidneys and spleen did not reveal lesions associated with any of the treatments.

DISCUSSION

This study was designed to determine if HI-6 in saline will produce lesions at the injection sites in muscles. If it does, was the duration of lesions dose-dependent? The dosages used were based on the therapeutic dosages against GD (Lipp and Dola, 1980; Shaw et al., 1981). Lipp and Dola reported that 30 mg/kg of HI-6 was therapeutic against 60 µg/kg of GD in 1 of 2 monkeys. Shaw and coworkers (1981) showed that 125 mg/kg of HI-6 was therapeutic against 2 to 8 LD_{50} of GD in rats, or 5 LD_{50} of GD in guinea pigs. From this information, the 200 mg/kg dose was chosen and deemed useful as a high-level dose for use in the safety-in-use evaluation of HI-6. This dose and the solubility limits of HI-6 in saline (280 mg/mL), at ambient temperature, necessitated the use of a 0.8 mL/kg inoculum. In unpublished studies, 0 to 415 mg/mL of HI-6 (0 to ≈3320 mg/kg), in 1-8 mL inocula, were used in the rat (Bier, 1985a) and 93 to 588 mg/mL of HI-6 (9.3 to 4650 mg/kg), also in 1-8 mL inocula, used in the dog (Bier, 1985b).

The pH of HI-6 solutions was not adjusted because we wanted to confine the study to the effects of HI-6 in saline without additional chemicals. At pH higher than that used in this study, HI-6 has been shown to degrade rapidly (unpublished reports, W.D. Marshall, et al., 1986; W.D. Marshall and F.M. Fouad, 1987).

Saline was used as a medium for HI-6 because of its acceptable physiological properties. Nevertheless, it is evident that it too produced a reaction when injected into muscle tissues. Haemorrhage and congestion of the early lesion may also have been associated with trauma caused by the needle puncture at the time of injection. The tissue reaction with HI-6 on Day 3 was qualitatively similar to that associated with saline. A research project addressing the injection site complications of drug therapy is underway under the scientific authority of P. Angus and M.-C. Belanger, Alberta Mental Health Services (Alberta Foundation for Nursing, 1987).

The severity and persistence of lesions were dependent on the HI-6 dose given to animals. All muscles injected with 200 mg/kg of HI-6 had lesions on Days 3, 7 and 14, whereas muscles injected with 50 mg/kg had mild macroscopic lesions on Day 3 only.

The lymph node necrosis seen in animals receiving the high dose (200 mg/kg) of HI-6 is noteworthy but its etiology is undetermined and may even be unrelated to HI-6. This lesion, therefore, requires further investigation.

Our results concur with unpublished reports that the severity of muscle lesions in the injection sites of rats (Bier, 1985a) and dogs (Bier, 1985b) was dependent on the dose of HI-6. On Day 14, Bier (1985a) did not see microscopic lesions in rats injected with either

distilled water (control) or HI-6 at 83, 174 and 363 mg/kg. There was mild or moderate acute degeneration of muscle fibers at higher doses. However, slight to moderate lesions were seen in some dogs injected with a single dose of HI-6 at the 147.2 mg/kg and even at the 9.3 mg/kg dose in addition to severe lesions seen in the 293.7 to 588.9 mg/kg groups (Bier, 1985b).

The results suggest that lesions due to injection of HI-6 eventually healed but that the severity and persistence of necrosis were dependent on the concentration of HI-6 injected. Lesions produced by HI-6 at a therapeutic dose level of 50 mg/kg healed completely. Furthermore, on Day 3 the sites injected with the saline solution also showed haemorrhages and/or congestion, which could not be differentiated from lesions caused by HI-6. However, these lesions were transient and healed completely in a few days unlike those produced by HI-6 at 200 mg/kg.

CONCLUSION

Histopathological examinations suggested that the severity and the rate of remission of the injection site lesions due to HI-6 were dose dependent. Lesions in muscles injected with 50 mg/kg of HI-6 healed within 7 days whereas those due 200 mg/kg were still prominent but showing signs of remission by Day 14. Although the implications of these lesions in terms of human performance and long-term effects are not known, these results indicate that HI-6, injected as proposed human therapeutic dose levels, will not cause lasting muscle damage.

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TABLE 1

THE HI-6 DOSE AND NUMBER OF ANIMALS USED PER TREATMENT GROUP

HI-6 in Saline (mg/kg)	No. of Animals	No of Animals Killed		
		Day 3	Day 7	Day 14
0	6	2	2	2
50	15	5	5	5
200	15	5	5	5

TABLE 2

PREVALENCE OF PATHOLOGICAL CHANGES IN MUSCLE AND REGIONAL LYMPH NODES OF RABBITS TREATED WITH SALINE OR HI-6

		Macroscopic	Microscopic	
HI-6 (mg/kg)	Day of Kill	Muscle	Muscle	Node
0	3	6/14	7/13*	0/11*
	7	1/14	1/14	3/14
	14	0/13	0/13*	3/12*
50	3	5/5	5/5	0/5
	7	0/5	2/5	0/4
	14	0/4	0/4	0/4
200	3	5/5	5/5	0/1*
	7	4/5	5/5	1/5
	14	3/5	5/5	4/5

* Low denominator means that some samples were not examined due to sampling problems.

TABLE 3

MEASUREMENTS (CM) OF MACROSCOPIC LESIONS IN GASTROCNEMIUS MUSCLES OF MALE RABBITS INJECTED WITH SALINE OR HI-6

HI-6 (mg/kg)	Day of Kill	Lesion Diameter*	
		Saline (n)	HI-6 (n)
0	3	0.05 ± 0.05 (4)	-
	7	0.00 ± 0.00 (4)	-
	14	0.00 ± 0.00 (4)	-
50	3	0.08 ± 0.05 (5)	0.10 ± 0.00 (5)[b]
	7	0.00 ± 0.00 (5)	0.00 ± 0.00 (5)[c]
	14	0.00 ± 0.00 (4)	0.00 ± 0.00 (4)
200	3	0.38 ± 0.28 (5)[a]	2.30 ± 0.44 (5)[a,b]
	7	0.20 ± 0.20 (5)	1.50 ± 0.39 (5)[a,c]
	14	0.00 ± 0.00 (5)	0.56 ± 0.31 (5)

* Mild, diffuse and barely visible lesions were assigned an arbitrary value of 0.2 cm for calculation purposes. Means with the same alphabet superscripts are significantly different from each other ($p \leq 0.01$). The difference between the saline groups Day 3 and Day 7 was significant at $P \leq 0.05$.

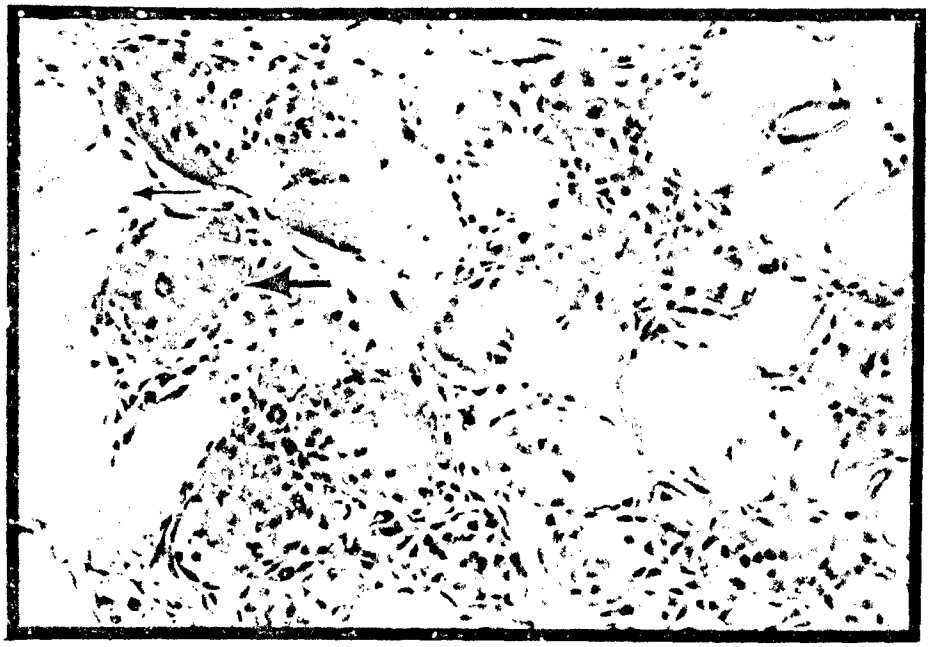

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Figure 1

Changes in gastrocnemius muscle of a rabbit injected with saline 3 days previously. Note normal muscle fibers on the left (small arrow) and necrotic fibers (large arrow) within the area of tissue reaction which also includes numerous macrophages and mononuclear cells but very few polymorphonuclear cells. (HE, 200 ×)

Figure 2

Changes in gastrocnemius muscle of a rabbit injected with 50 mg/kg of HI-6 in saline 3 days previously. Note normal muscle fibers (small arrow) on the right and necrotic fibers (large arrow) on the left. A tissue reaction at the center of these necrotic fibers is similar to that shown in Fig. 1. (HE, 200 ×)

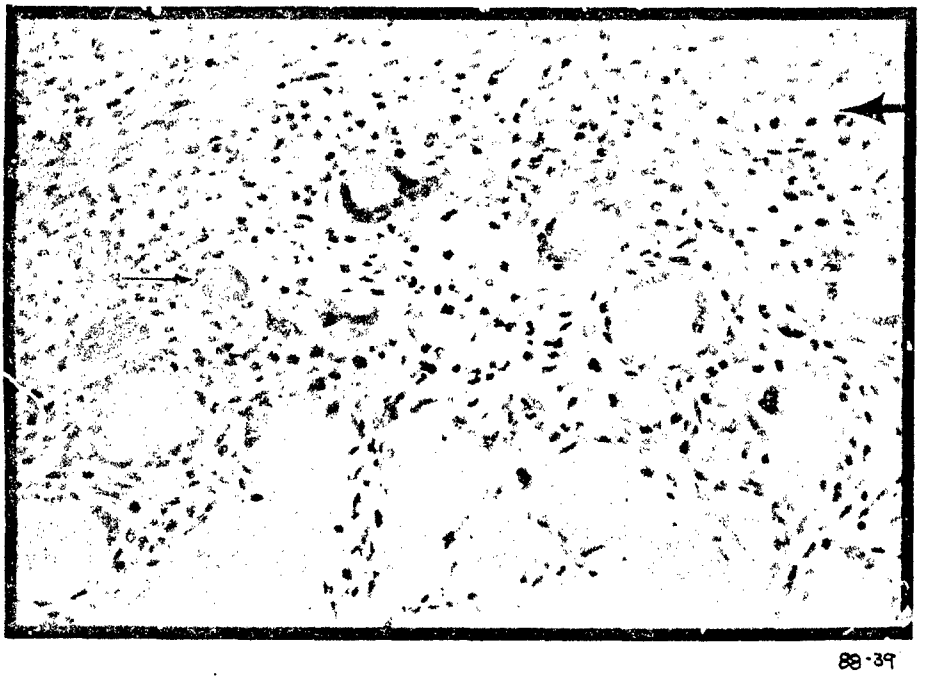

Figure 3

Changes in gastrocnemius muscle of a rabbit injected with 200 mg/kg of HI-6 in saline 14 days previously. Various stages of myophagia, suggestions of giant cell formation (small arrow), and fibroblast-rich connective tissue (large arrow) are shown. (HE, 200 ×)

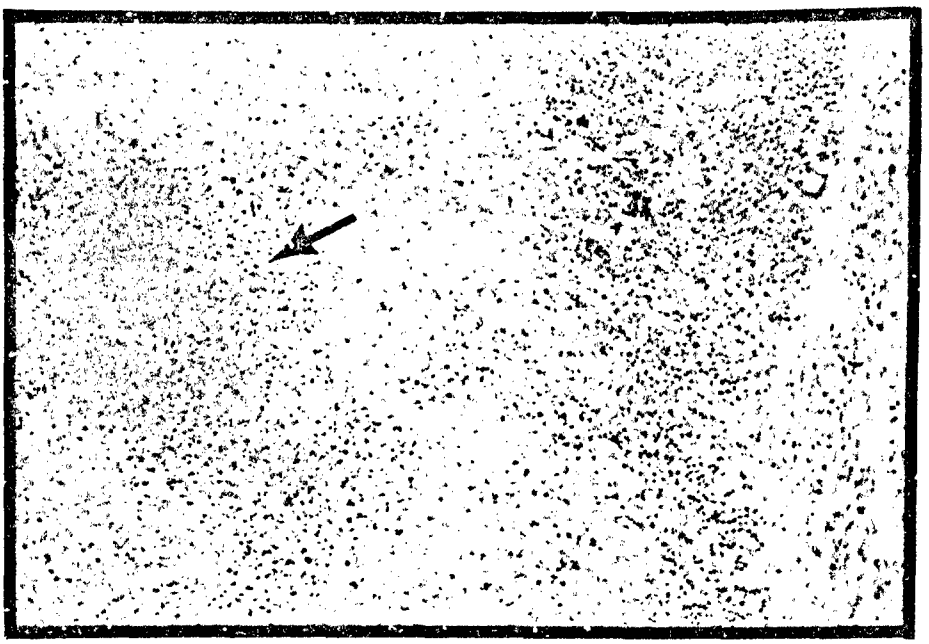

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Figure 4

Focal necrosis (arrow) in the cortex of the lymph node which received lymph from muscle injected with 200 mg/kg of HI-6 in saline 7 days previously. Note moderate numbers of polymorphonuclear cells in the tissue on the right side of the figure. (HE, 100 ×)

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